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14. ABSTRACT Interleukin-6 (IL-6) is a key signaling molecule in breast cancer cells. It is responsible for many cellular responses in both cancer and normal cells, including immune response, cell survival, cell death, and proliferation. Unfortunately, IL-6 may also play a key role in the progression of breast cancer from stage I to stage IV cancer (typically associated with a poor prognosis among breast cancer patients). This change to a more serious cancer is associated with significantly increased levels of IL-6, which is believed to affect the subsequent proliferation and metastasis of the tumor cells by initiating a complex series of molecular signal pathways, specifically the IL-6/JAK2/STAT3 pathway. Therefore, we are examining a new strategy to combat breast cancers by disrupting the initiation of the IL-6 signaling using small synthetic molecules using the natural product madindoline A as a starting point for our studies. Madindoline A (MDL-A) is known to interact with the IL-6 receptor on the surface of the cell and prevent this signaling event. Modification of the chemical structure of MDL-A and new design using it as a structural template should provide more potent and selective derivatives which may be useful therapeutic agents for the treatment of breast cancer. Thus, a multidisciplinary team has been assembled with expertise in computational chemistry, synthetic chemistry and cancer biology in order to design and synthesize the new compounds, and in biochemical and cellular assays to assess the effectiveness of these agents. To date, more than twenty novel analogues have been synthesized and partially tested for their ability to bind to gp130 and inhibit STAT3 phosphorylation. The data obtained during the course of our studies into the anticancer properties of these molecules will be utilized to refine and improve upon our model and our synthetic analogues with the ultimate goal of developing a useful treatment for late stage breast cancers.

15. SUBJECT TERMS
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INTRODUCTION

Interleukin-6 (IL-6) is a key signaling molecule in breast cancer cells. It is responsible for many cellular responses in both cancer and normal cells, including immune response, cell survival, cell death, and proliferation. Unfortunately, IL-6 may also play a key role in the progression of breast cancer from stage I to stage IV cancer (typically associated with a poor prognosis among breast cancer patients). This change to a more serious cancer is associated with significantly increased levels of IL-6, which is believed to affect the subsequent proliferation and metastasis of the tumor cells by initiating a complex series of molecular signal pathways, specifically the IL-6/JAK2/STAT3 pathway. Therefore, we are examining a new strategy to combat breast cancers by disrupting the initiation of the IL-6 signaling using small synthetic molecules using the natural product madindoline A as a starting point for our studies. Madindoline A (MDL-A) is known to interact with the IL-6 receptor on the surface of the cell and prevent this signaling event. Modification of the chemical structure of MDL-A and new design using it as a structural template should provide more potent and selective derivatives which may be useful therapeutic agents for the treatment of breast cancer. Thus, a multidisciplinary team has been assembled with expertise in computational chemistry, synthetic chemistry and cancer biology in order to design and synthesize the new compounds, and in biochemical and cellular assays to assess the effectiveness of these agents. To date, more than twenty novel analogues have been synthesized and partially tested for their ability to bind to gp130 and inhibit STAT3 phosphorylation. The data obtained during the course of our studies into the anticancer properties of these molecules will be utilized to refine and improve upon our model and our synthetic analogues with the ultimate goal of developing a useful treatment for late stage breast cancers.

BODY

Task 1. Design and synthesize novel madindoline A analogues as inhibitors of the IL-6/GP130 interaction for the treatment of breast cancer.

1. *Computational design and optimization will be carried out throughout the entire project period. (Months 1-24)*

PART ONE

In order to examine the relative binding positions and poses of MDL-A and the novel analogues MDL-5 and MDL-16 (an improved MDL-5 analog for both potency and synthesis), a docking study was carried out. Based on this study, it was determined that both MDL-5 and MDL-16 bind with similar binding conformations as MDL-A (Figure 1D) and do pick up the additional interactions predicted from the extra subpockets in the gp130 D1-domain (see Figure 1C). As a result, the predicted binding energies of the designed analogues were better than MDL-A. MDL-5 and MDL-16 both showed about a -3 kcal/mol improvement in binding energy compared to the MDL-A binding energy. 1.36 kcal/mol difference in binding energies corresponds to 10 fold difference in activity, which is close to the difference in activity observed

experimentally between MDL-5 and MDL-A. It should be noted, however, that there is a statistical error associated with these docking studies of approximately 2 kcal/mol.

The relative stability of the three compounds in the MDL-A binding pocket has also been examined. The binding stabilities of MDL-A, MDL-5 and MDL-16 are represented in atomic fluctuations with respect to their initial binding conformation (Figure 2). Atomic fluctuations were calculated by averaging atomic fluctuations over 20 ns MD simulation. The HFI unit of MDL-A showed instability in the gp130 D1-domain binding pocket, whereas the hydrophobic tail (diketo cyclopentedene ring) of MDL-A showed stability with least atomic fluctuations. The southern tail of MDL-A seems to be very important and keeps it from "flying away" from the binding pocket indicating a more significant interaction. Our computational results are consistent with the previous study which showed that the tail portion of MDL-A is important for its activity and supported the results of Saleh et al.[1], which demonstrated that a compound containing only an HFI unit was not capable of inhibiting gp130 homodimerization. Our MDLs MD simulation studies showed consistent results. MDL-5 and MDL-16 showed very stable confirmation at D1-domain binding pocket (Figure 2). Average root mean square fluctuations (RMSf) for MDL-16, MDL-5 and MDL-A were 1.6 (±0.4), 1.7 (±0.4) and 4.0 (±1.1), respectively. Stable binding dynamics of MDL-5 and MDL-16 validated our design idea and predicted that these compounds should demonstrate improved activity. In order to calculate the absolute binding energy of MDL-A, MDL-5 and MDL-16, we calculated binding free energy of each complex using MMPBSA methods.

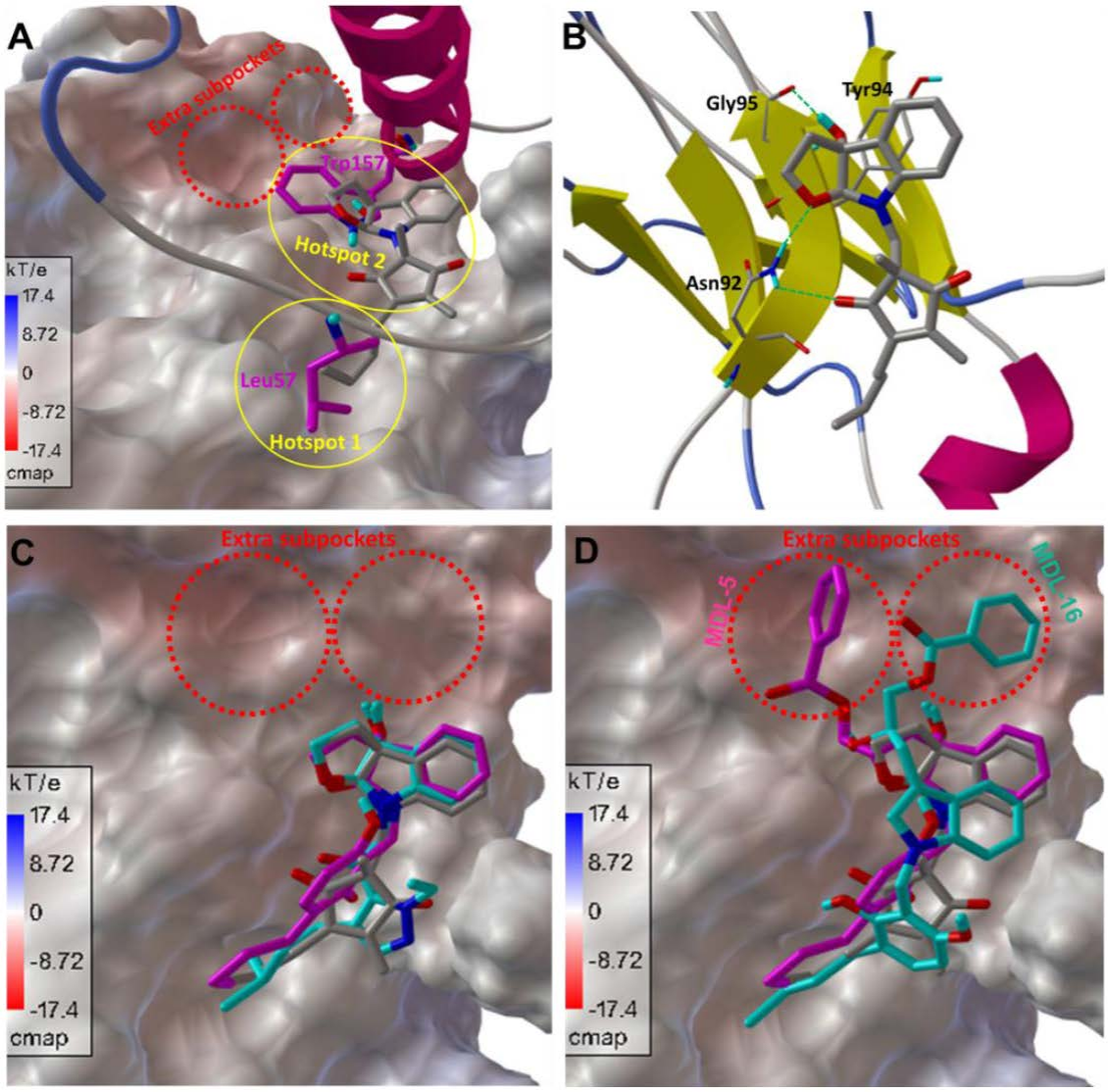


Figure 1. Binding modes of designed MDL-A analogues: A. gp130 D1-domain in electrostatic surface representation; IL-6 in ribbon representation. The two larger yellow ellipses indicate two major binding "hot spots" between IL-6 and gp130. The small dotted red circle points to an empty polar extra subpockets. **B.** D1 domain in ribbon representation and MDL-A in thick ball-and-stick. **C.** Modified southern pentendione ring of MDL-A: Overlaid binding modes of MDL-A (grey color), pyrazole analogue (cyan color) and hydroxyl analogues (magenta color) are shown in ball and stick representation on gp130 D1-domain surface; **D.** Binding modes of MDL-5 (magenta color) and MDL-16 (cyan color) are shown overlaid with MDL-A on gp130 D1-domain. MDL-5 and MDL-16 captures additional binding interactions from extra subpockets shown as small dotted red circles.

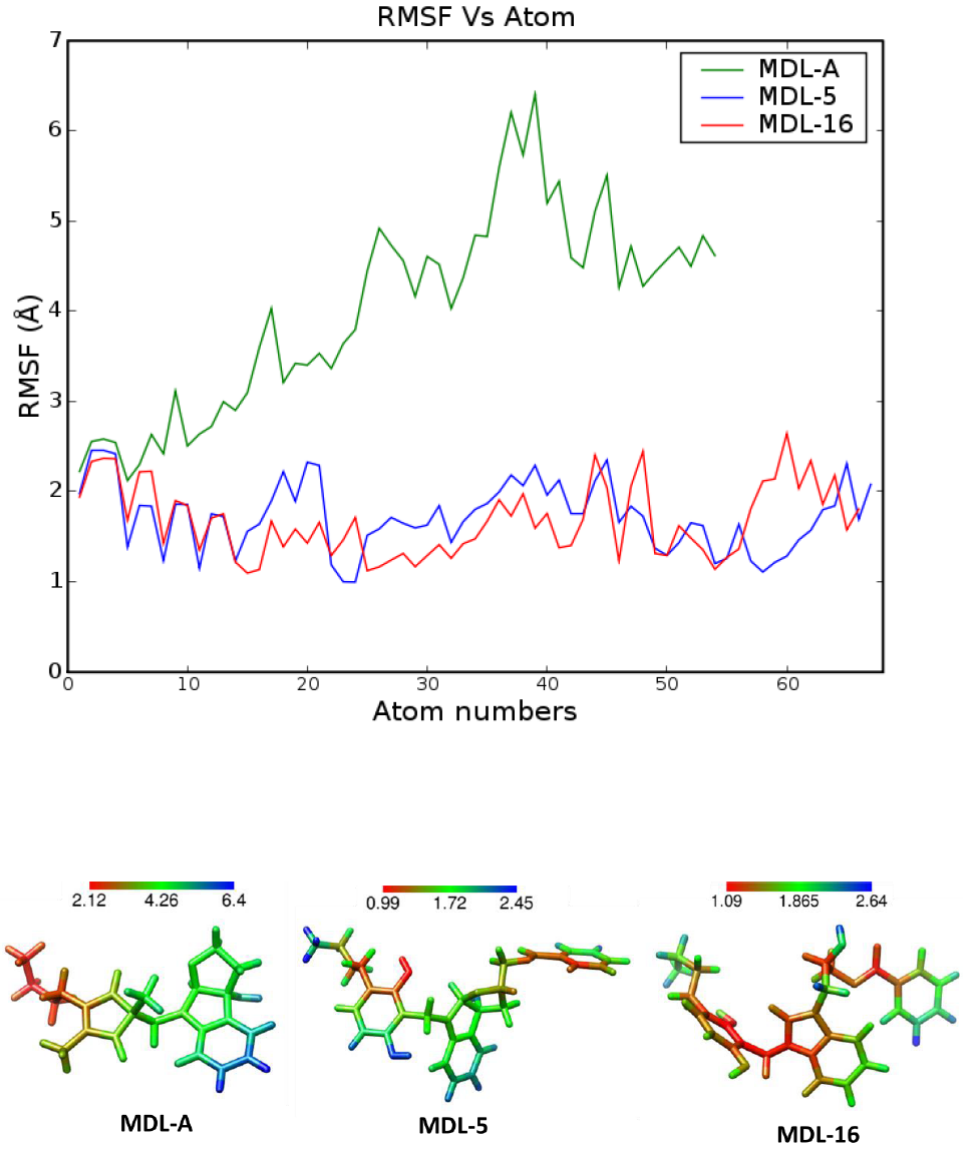


Figure 2. Atomic fluctuations of MDL-A, MDL-5 and MDL-16 during a 20 ns MD simulation. MDL-16 (red color RMSf) and MDL-5 (blue color RMSf) showed stability at the binding pocket compared to MDL-A (green color RMSf). Atoms are colored based upon their RMSf (Root mean square fluctuations).

The results of the stability analysis were confirmed by looking at a per residue free energy calculation. The binding free energy contributions of each amino acid residues of D1-domain were calculated for each complex (IL-6/D1-domain, MDL-A/D1-domain, MDL-5/D1-domain and MDL-16/D1-domain). All complexes showed overlap in amino acid residues which are involved in binding interactions and contribute to the overall bind free energies (Figure 3). For the MDL-A/D1-domain complex, amino acid residues which interact with the tail of MDL-A contribute most towards binding

free energy. For MDL-5 and MDL-16 additional free energy was gained by interactions with the extra subpocket amino acid residues.

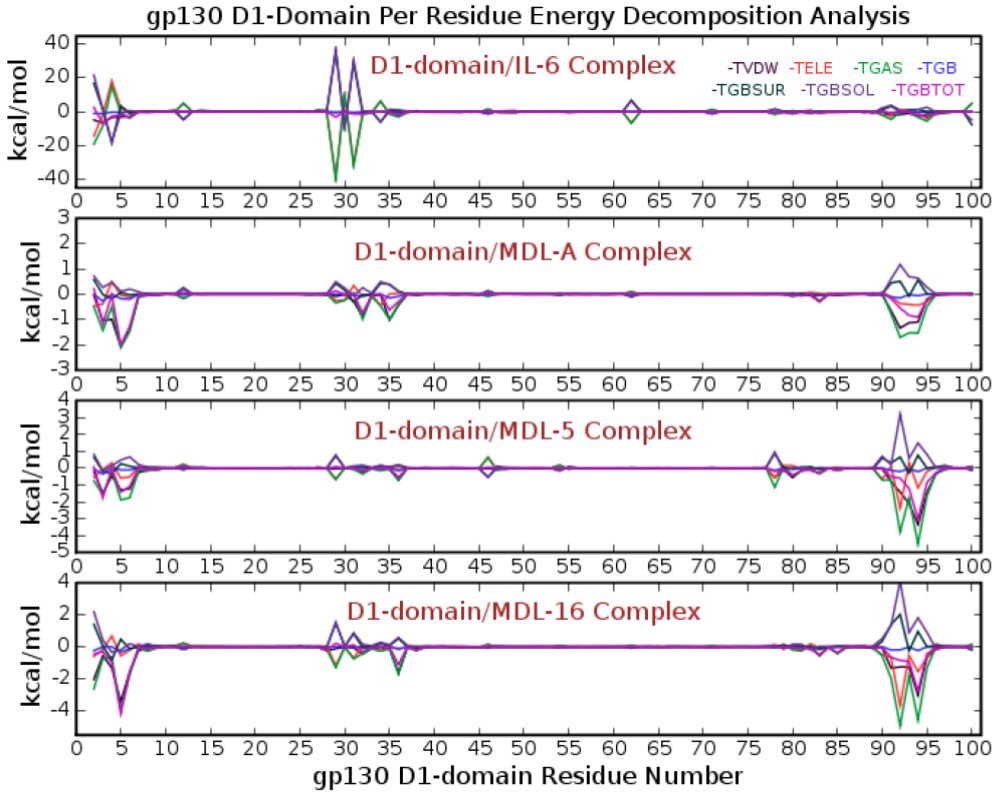


Figure 3. Binding free energy decomposition on per residue contributions for IL-6/D1-domain complex, MDL-A/D1-domain complex, MDL-5/D1-domain complex and MDL-16/D1-domain complex: *EVDW*: van der Waals energy component; *EELE*: Electrostatic energy component; *EGAS*: Gas phase energy component (*EVDW* + *EELE* + *Eint*); GGB: electrostatic solvation energy using GB model (polar contribution); *GGBSUR*: nonelectrostatic solvation components (nonpolar contributions); *GGBSOL*: Solvation free energy component; *GGBTOT*: (*EGAS*+*GGBSOL*).

An analysis of each of the forces responsible for the binding interactions of MDL-A, MDL-5, and MDL-16 has also been carried out by examining the enthalpy *(ΔH)* and entropy *(ΔS)* of the system, the thermodynamic forces responsible for binding free energies $(\Delta G_{binding})$. By utilizing the additive nature of the enthalpy term in the binding free energy calculation, each component was separated into polar, nonpolar, electrostatic and hydrophobic terms. The results (not shown) demonstrated that enthalpy was the driving force for the binding interactions of MDL-16, MDL-5 and MDL-A to the gp130 D1-domain. For all compounds hydrophobic and nonpolar interactions were dominant forces, particularly van der Waals forces. The nonpolar term ($G_{nonpolar}$) of solvation free energy was favorable while the polar term (G_{polar}) was unfavorable for complex formation in all cases. Calculated binding free energies show that MDL-16 is more potent than MDL-5 and which in turn is better than MDL-A.

Milestone 1: we have discovered MDL-16, the most potent small molecule IL-6 inhibitor so far.

PART TWO

In recently years, my lab has developed a novel ligand/protein simulation method called Multiple Ligand Simultaneous Docking (MLSD) [2]. We used it for fragment-based drug design

and drug repositioning/repurposing, a popular drug discovery strategy nowadays (existing drugs for novel targets). We successfully found that anti-inflammatory drug, celecoxib, is a weak inhibitor of STAT3 oncoprotein [3]. In a similar fashion, we have tried it on the D1-domain here.

We built a small library of feature fragments from key interacting residues (Leu57 and Trp157) of IL-6, inhibitor MDL-A and the more potent analogues MDL-5 and MDL-16. The feature fragments are listed in Figure 4. Here the aromatic indole fragment is a key moiety to mimic residue Trp157 of IL-6, and the ButylPhenyl fragment is used to displace the hydrophobic Leu57 of IL-6. Learning from the hot spot binding residues of IL-6 and the feature fragments of inhibitor MDL-A, our strategy is to identify drug scaffolds with stronger affinities. To avoid fragments with undesired drug ADMET properties, drug scaffolds structurally or chemically similar to the feature fragments were identified by sub structure or similarity searches on a drug scaffold database and DrugBank. Figure 5 lists the drug scaffolds identified, which were grouped into 2 pools: aromatic and nonpolar. The aromatic scaffolds in pool 1 favor binding to the Trp157 site, and the nonpolar scaffolds in pool 2 are for the Leu57 site or the extra subpockets. Piperidine and cyclohexane, very common six member rings in drugs, were used to replace the aliphatic tail of MDL-A to improve the binding affinity for the deep hydrophobic Leu57 binding pocket on the gp130 D1 domain.

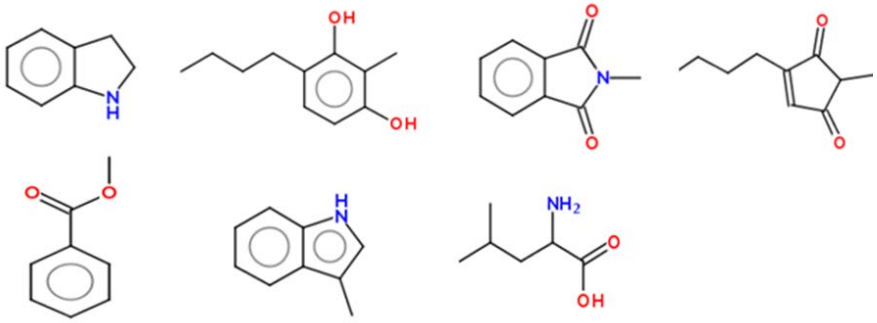


Figure 4. Feature fragments from Trp157 and Leu57 residue of IL-6, inhibitor MDL-A and its analogues to mimic the hot spot residues of IL-6.

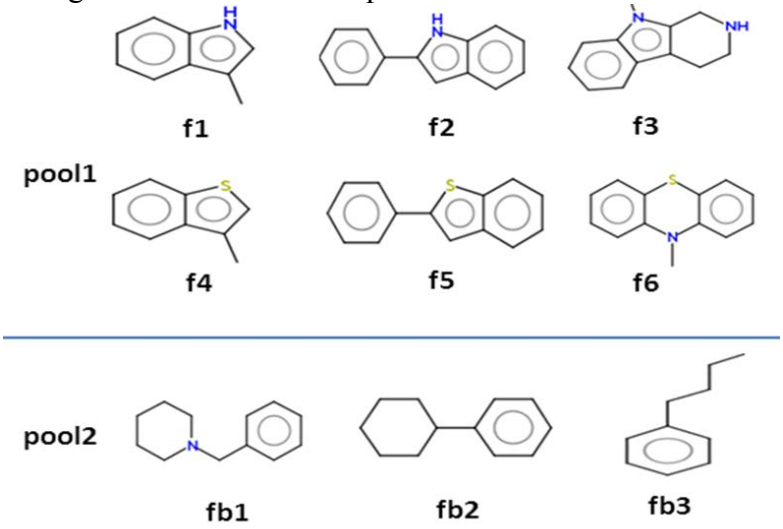


Figure 5. Structure of drug scaffolds identified for the binding hot spots of gp130.

To improve binding affinity, we applied MLSD to dock multiple drug scaffolds in a concerted way to the 2 binding hot spots of GP130, effectively disrupting multiple key residues of IL-6 binding to GP130 D1 domain. Briefly, the combinations of two drug fragments, one from pool 1 and the other from pool 2, were used as inputs for MLSD docking screening. Briefly, we found that f5/fb1 fragment combination is the most optimal. We linked this two fragments to

generate an *in silico* structural template and searched DrugBank, and found that Evista/ Raloxifene, an anti-osteoporosis drug, is also inhibit IL-6/gp130 integration. Figure 6 shows its binding to D1 domain.

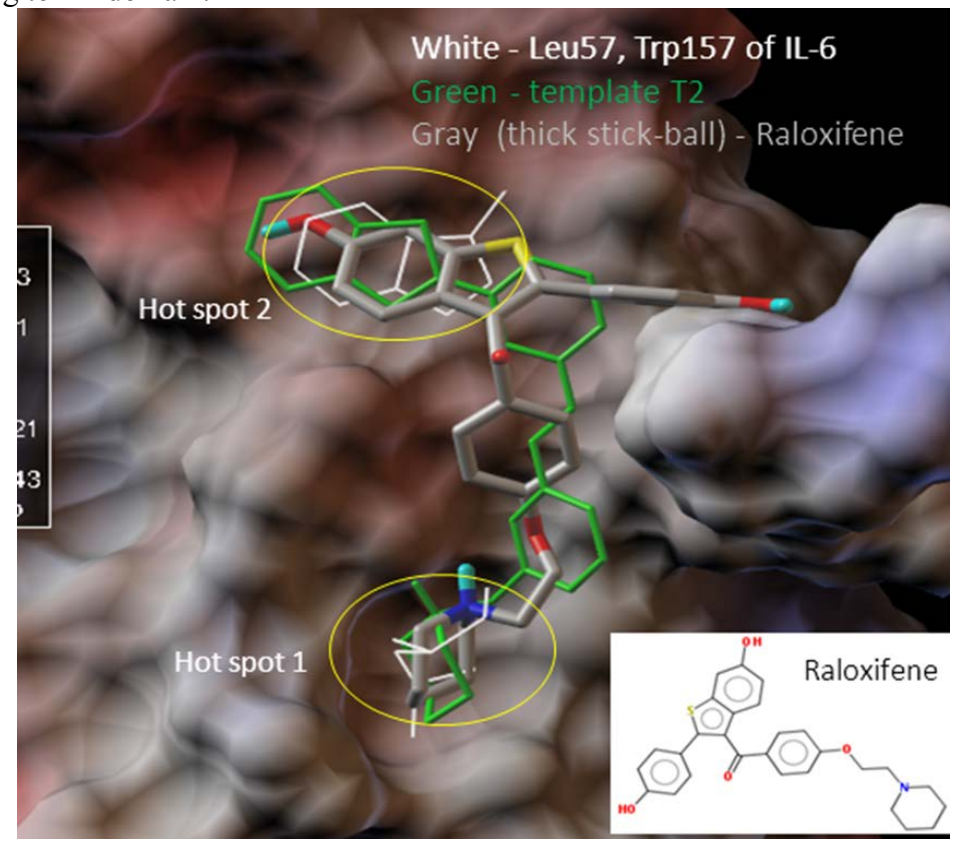


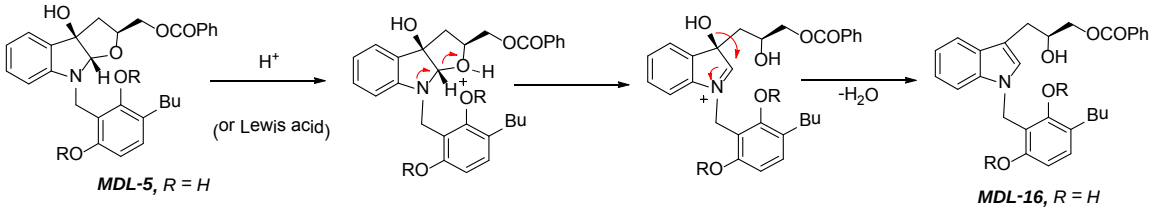
Figure 6. Raloxifene binding to the GP130 D1 domain to disable IL-6/GP130 interaction. T2 template is *in silico* molecule built from fragments f5/fb1. Both T2 and raloxifene disrupt key IL-6/GP130 hot spot interactions.

Milestone 2: We have discovered that anti-osteoporosis drug, Raloxifene, is an IL-6 inhibitor.

2. *Initial laboratory synthesis of madindoline A analogues for in vitro biological testing will be carried out. (Months 1-24)*

In the synthetic portion of the year 1 (2010-2011) report, we disclosed the synthesis of MDL-5, our most potent analogue with regard to direct gp130 binding activity. This compound contains a benzoyl side chain attached to the HFI unit which is designed to take advantage of interactions with the "additional" subpockets surrounding the MDL-A binding site. Unfortunately, relatively low yields of the product were produced through the alkylation and hydrogenation (deprotection) steps necessary to join the "northern" and "southern" halves of the molecule and reveal the free phenolic oxygens. In an effort to understand this low yield, we examined these reactions more closely. Although it appeared that the alkylation reaction proceeded very slowly, only starting material and product were found as major components in the reaction mixture. In the hydrogenation reaction, however, an unexpected byproduct was also isolated suggesting that the hemi-aminal moiety of the HFI unit is somewhat unstable. In this case, it appears to rapidly undergo a ring opening and elimination reaction in the presence of acidic or Lewis acidic reagents to produce the indole (or more appropriately the tryptophol unit)

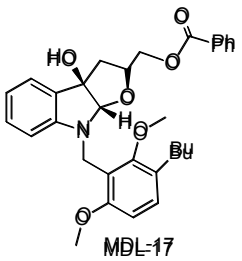
as the core of the northern half of the molecule as shown in Scheme 1. This new compound was assigned the code MDL-16. Interestingly, the tryptophol, which we had previously considered as a possible "bioisostere" of the HFI unit based on similar hydrogen bonding potential, overall size, and geometry, was found to be slightly more active than MDL-5 itself in the gp130 binding assay (see Task 2 below). In addition, the ring opening does not appear to be as significant in systems that lack the additional benzoyl substituent found in MDL-5, suggesting that the HFI unit in MDL-A may be somewhat more stable that of MDL-5. At this stage, however, direct comparison of these ring systems has not been rigorously examined.



***MDL-5**, R = H* ***MDL-16**, R = H*

Scheme 1. Proposed mechanism for the ring opening transformation of the HFI unit to the indole in the presence of acid.

In an effort to prevent the formation of the MDL-16 byproduct and improve the yields of MDL-5 or related compounds, a number of alternative protecting group strategies were explored. These included using the MOM (methoxymethyl) groups and Me groups explored during the initial synthesis of the benzyl subunit (Scheme 2, year 1 report). In both of these cases, however, these attempts provided little to no improvement over the hydrogenation as MDL-16 was also formed at the expense of MDL-5 during these deprotection reactions. For example, a variety of conditions attempted for the removal of the MOM groups is included in Table 1. These efforts did, however, provide us with an opportunity to make the MDL-5 analogue containing the dimethoxy substituted benzyl ring (MDL-17, right) in order to look at the effect that substitution of the phenols has on the ability to inhibit STAT3 phosphorylation. This compound is able to be generated in much larger quantities than MDL-5.

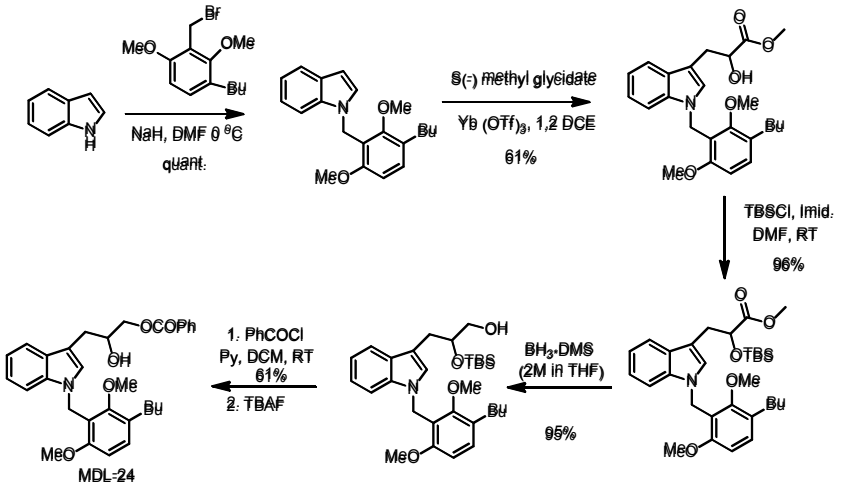


MDL-17

Table 1. Experimental conditions employed in an effort to remove the MOM protecting groups in the synthesis of MDL-5.

Sl .no	Reaction conditions	Results
1.	10% methanolic HCl	10% yield, multiple spot and base line material
2.	5% methanolic HCl	Deproctected only TBS group; MOMs were intact
3.	Ethylene glycol, 140 °C, 48 h	Very slow.
4.	*n*-PrSH, $ZnBr_2$, RT 5 min	Unknown products
	n-PrSH, $ZnBr_2$, 0 °C, 30 min	10% yield, Mixture of products(only deprotected TBS group + only one MOM group)
5.	*n*-PrSH, $ZnBr_2$, -15 °C, 3 h	15 % yield, Mixture of products(only deprotected TBS group + only one MOM group) and these were difficult to separate
6.	TMSBr, -78 °C to 0 °C 4h	Multiple spots
7.	PPTS, *n*- Butanone	Mostly baseline material
9.	PTSA.H_2O/Toluene	Multiple spots

Unable to effectively prevent the synthesis of MDL-16, we decided to embrace the formation of the product after discovering its potency relative to MDL-5. A thorough computational study was initiated to look at the relative binding conformations and energies of MDL-A, MDL-5, and MDL-16 to gp130 (previously described). Confident that this material could be synthesized more efficiently than MDL-5, we set out to optimize its synthesis and explore the structure-activity relationship of this novel indole class of compounds. Although this deviates slightly from the initial series of compounds proposed in the application, the lead identified through experimentation shows significant promise and should be able to more effectively be modified through synthetic manipulation than MDL-5. It is also expected to demonstrate increased stability. As anticipated, the indole core can rapidly and efficiently be functionalized to provide the methoxy protected MDL-16 derivative MDL-24 as shown in Scheme 2. Surprisingly, low yields are still obtained upon deprotection of the phenolic protecting groups in molecules of this type. This may be due in part to the application of acidic conditions in the presence of the indole and may be resulting in cyclization of an alcohol (or phenol) onto the indolenine generated via protonation of the 3 position of the indole. In this case, we have yet been unable to identify any byproducts of the reaction to confirm or deny this hypothesis. A literature search, however, revealed no similarly substituted benzylic indoles, indicating that reactivity and stability of this type of compound has not yet been reported.



Scheme 2. Synthesis of MDL-24 via modified route.

Regardless, a series of analogues has been synthesized using this approach. In addition to the dimethyl ethers (i.e. MDL-24), these compounds were designed to examine the importance of various functional groups in the MDL-16 molecule, including hydrogen bonding in the benzylic ring (MDL-18, -28, -29, and -30), the role of the hydroxyl substituent on the indole C3 chain (MDL-21 and -22), extension of chain length (MDL-23), the ability to prepare more hydrolytically stable compounds (MDL-19, -20 -25, -26, and -27), and the impact of benzoyl substitution. Attempts to synthesize compounds containing an ether linkage to the aryl ring on the C3 chain have not yet been successful.

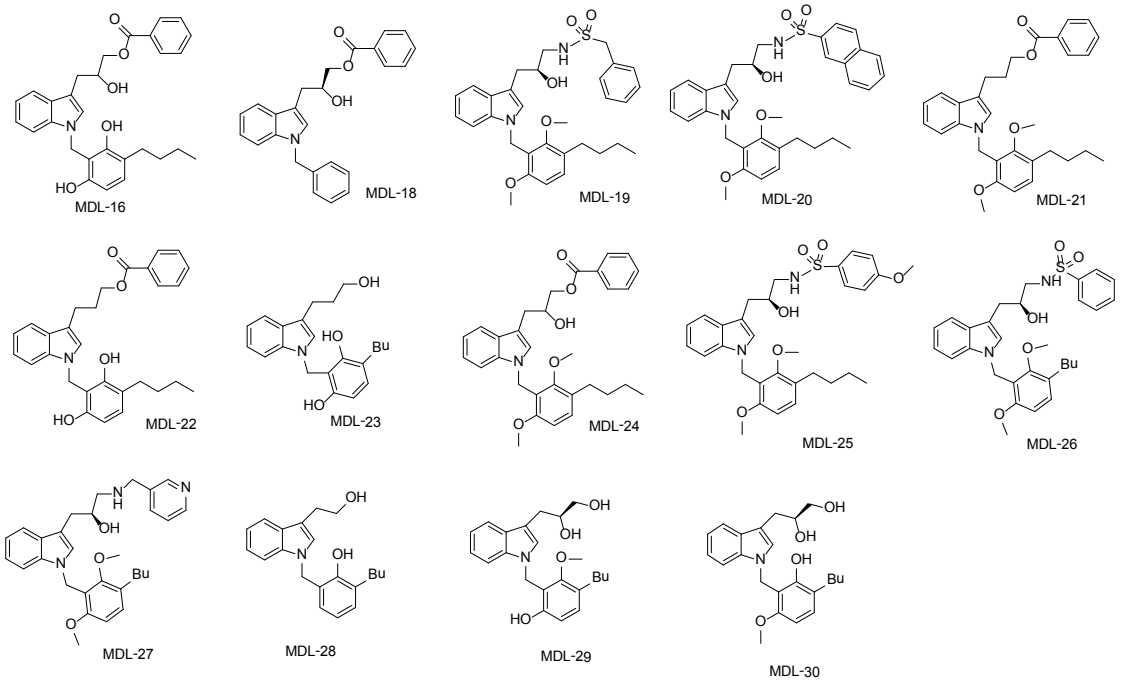


Figure 7. MDL-16 and structurally similar analogues.

Finally**,** attempts to synthesize compounds containing a hydroxymethyl substituent at the C5 position of the indole in order to capture additional interactions with pockets on the benzene ring side of the indole have failed to provide the originally proposed products. In large part, this is due to the fact that a hydroxymethyl substituent at this position of an indole is prone to an elimination reaction due to the donation of the nitrogen lone pair. In our case, this intermediate appeared to be generated under a number of reaction conditions and was specifically observed during reactions with reducing agents which returned only the C5-methyl derivative derived from delivery of a hydride to the reactive intermediate with loss of the hydroxyl group. This hydrogen bond donor could still be installed; however, in this case it will require the addition of a second carbon atom between the alcohol and the indole ring.

Task 2. In vitro and in vivo studies of the proposed inhibitors.

PART ONE

In our year 1 (2010-2011) report, we described D1 domain protein purification and Biacore measurement of MDL-5 binding to D1 with K_D of 37μM. Here we report that MDL-16 is a little more potent with K_D of 29μM. Figure 8 shows that the potency order is MDL-16 > MDL-17 > MDL-5 >> MDL-A (natural product).

Figure 9 shows strong inhibitory effects of MDL-5/16 on MCF-7 breast cancer cell STAT3 activation upon IL-6 stimulation. Figure 10 shows MDL-16 selectivity as it inhibits IL-6 but not LIF induced STAT3 phosphorylation, using MCF-7 cell line for testing. Figure 11 shows that both MDL-5/16 do not inhibit STAT1 activation, a tumor suppressor, again using MCF-7 as testing cell line.

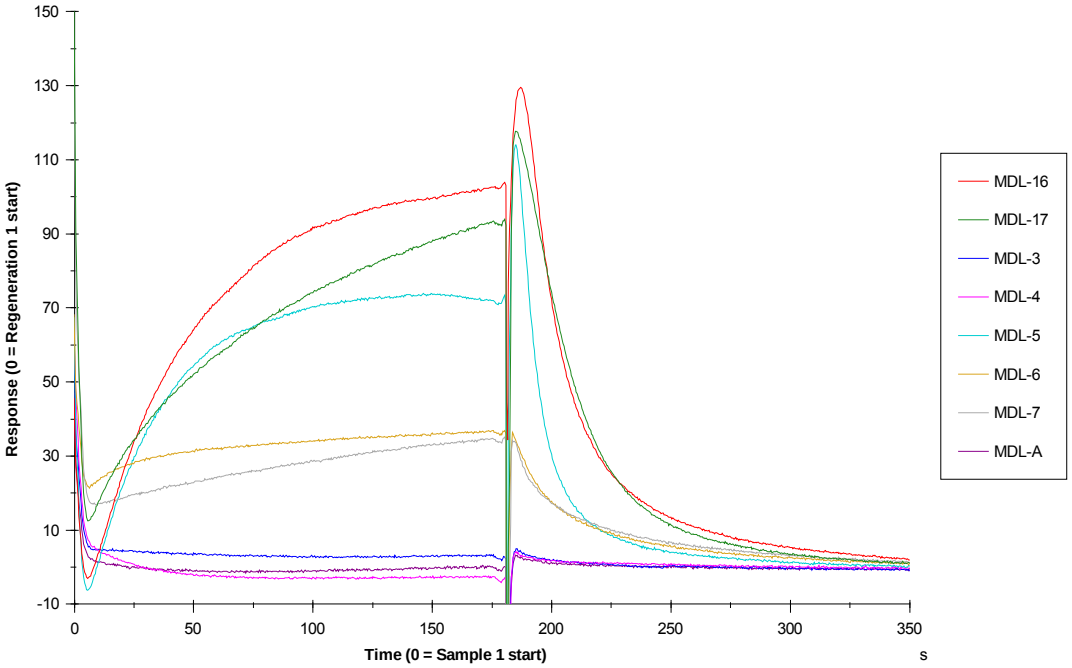


Figure 8. Biacore binding of MDL analogs to D1 domain of GP130.

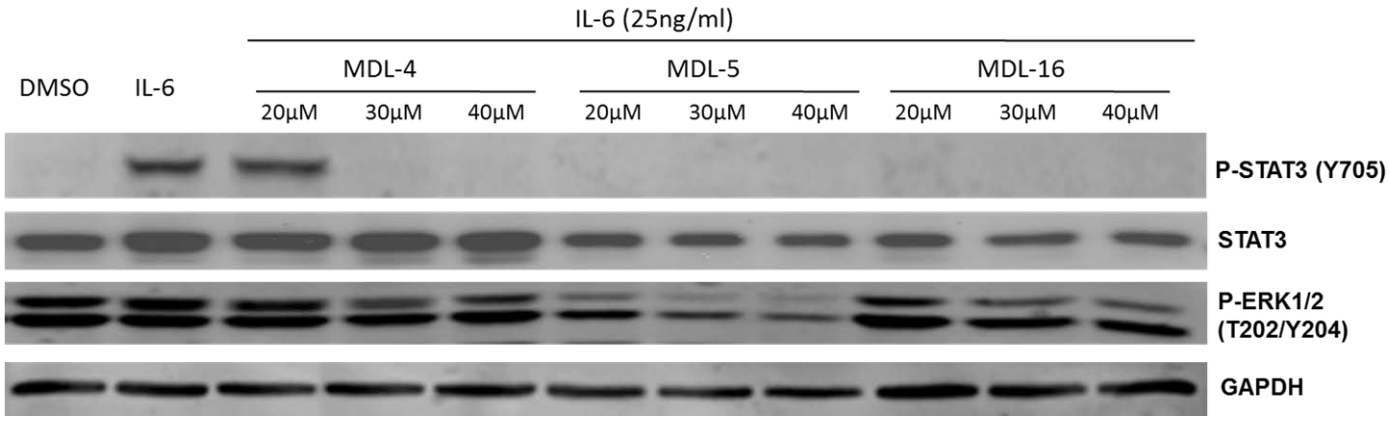


Figure 9. MDL-5/-16 suppress IL-6 stimulated STAT3 activation.

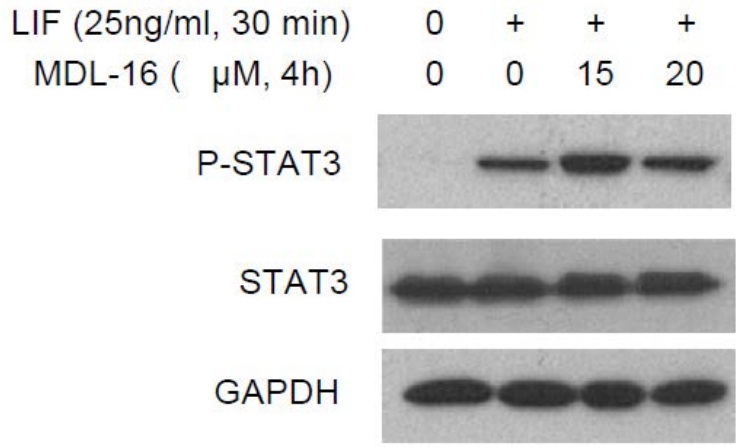


Figure 10. MDL-16 does not inhibit LIF induced STAT3 activation.

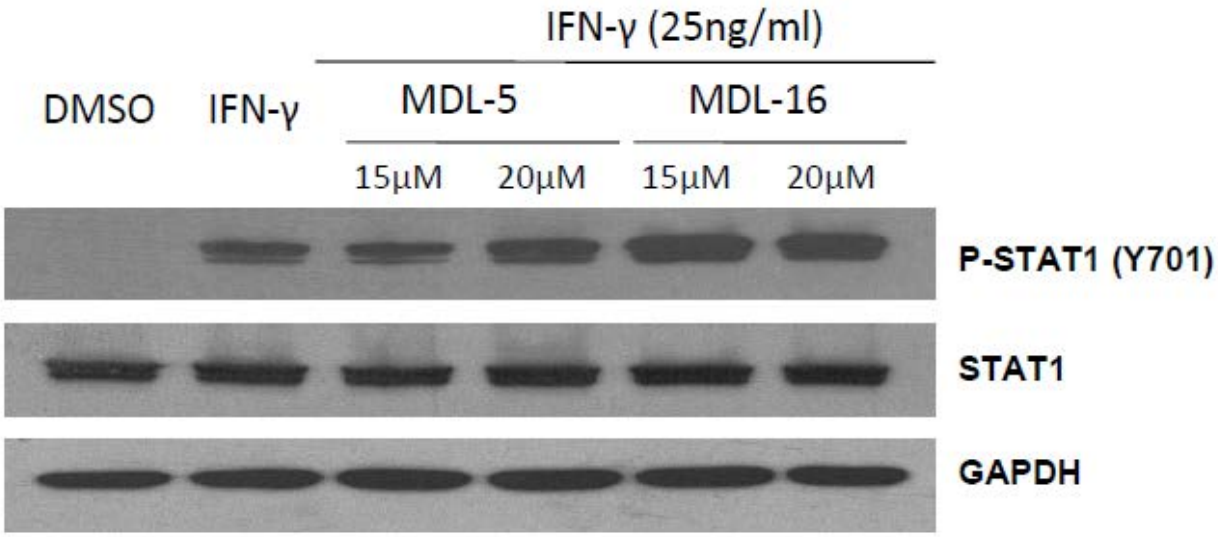


Figure 11. MDL-5/-16 do not affect STAT1 phosphorylation.

Our testings also show MDL-16 inhibits STAT3 nuclear translocation mediated by IL-6, as indicated in figure 12.

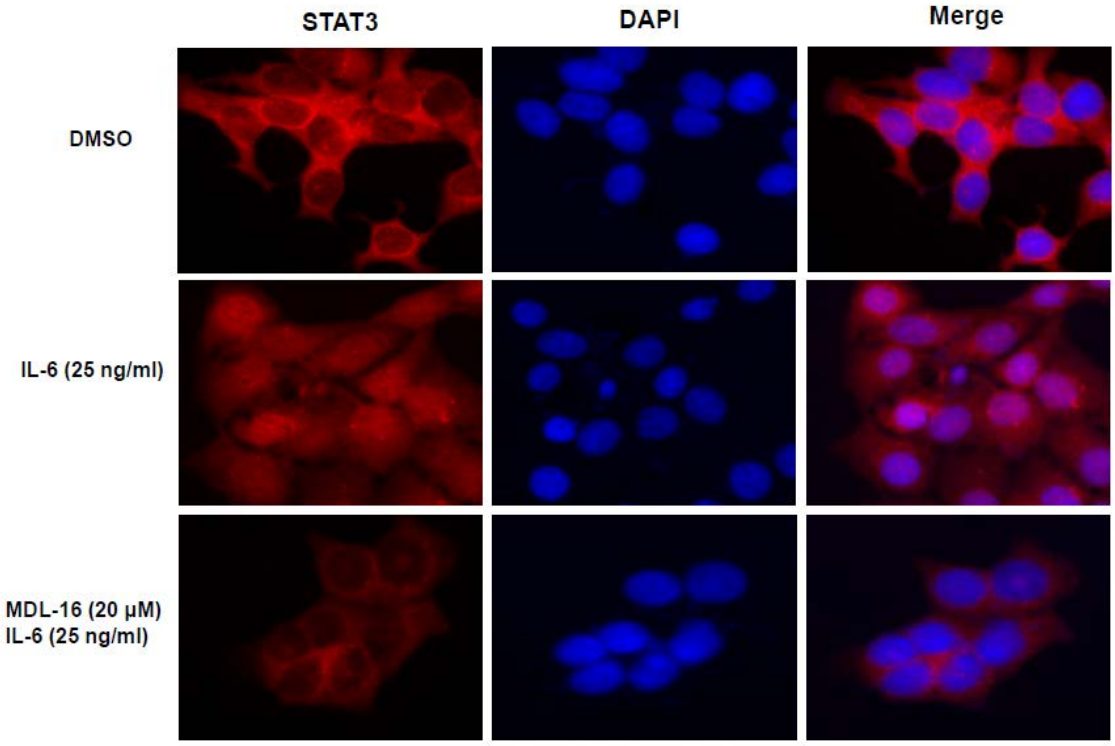


Figure 12. MDL-16 stops STAT3 nuclear translocation upon MCF-7 IL-6 stimulation.

PART TWO

We also tested Evista/Raloxifene IL-6 inhibition. Similarly, Evista shows IL-6 stimulated STAT3 inhibition, not STAT1 (figure 13) and its inhibition of STAT3 nuclear transcriptional activity (figure 14).

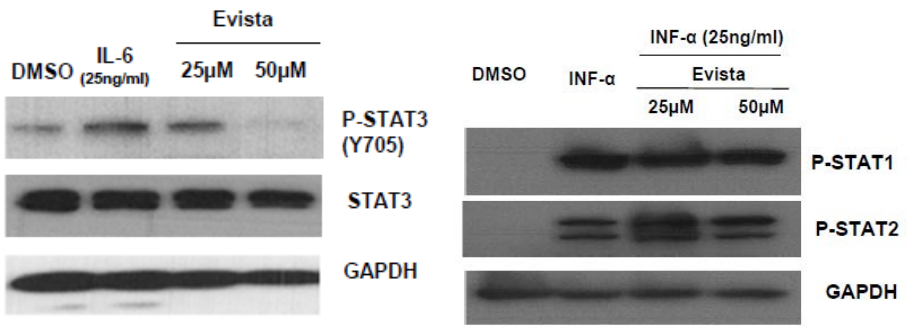


Figure 13. Evista inhibits IL-6 stimulated STAT3 activation, but not STAT1.

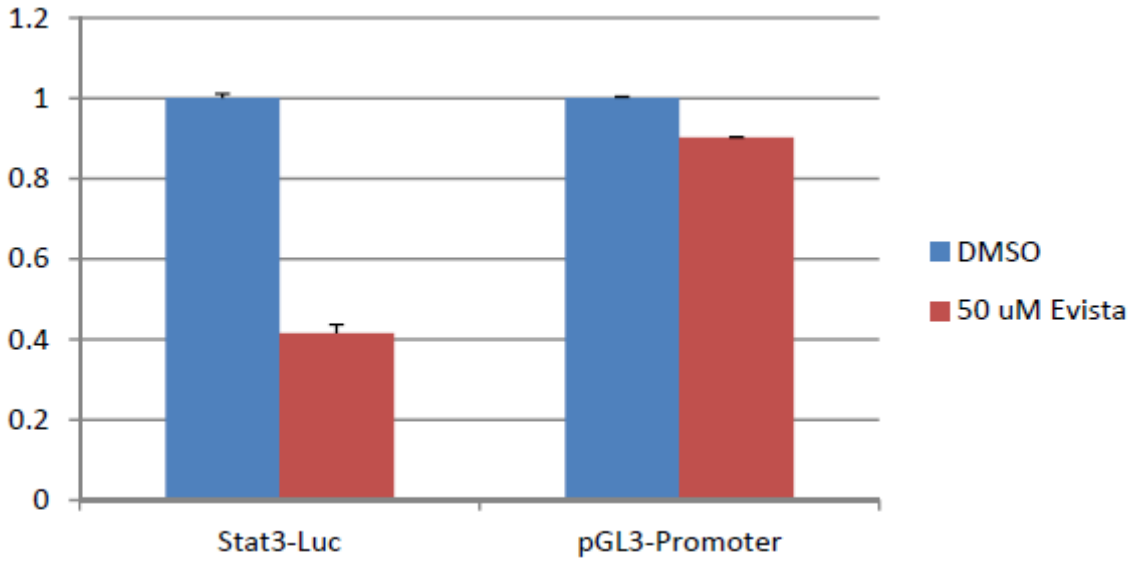


Figure 14. Evista inhibits STAT3 transcriptional luciferase activity, using Hela cell testing.

KEY RESEARCH ACCOMPLISHMENTS

- Our computational model elucidates that MDL-16 is more potent than MDL-5 through a stronger hydrophobic interaction; both compounds validate our design strategy on extra pocket binding besides the two "hot spots", resulting in much better inhibitors than MDL-A, the natural product.
- The binding free energy decomposition analysis gives insights on directions to improve the design further, especially the hydrogen-bonding to Asn92 and carbonyl of Leu79.
- An alternate synthesis of the "protected" MDL-16 system has been established which can be accomplished in 6 steps from indole.
- A total of 15 new analogues of MDL-A have been synthesized in year 2.

- Combining a novel docking method developed by us and fragment-based design, we found that the anti-osteoporosis drug, Evista/Raloxifene, is a novel IL-6 inhibitor. We successfully did "drug repositioning" on IL-6 inhibition design through computer modeling.
- Both MDL-16 and Raloxifene show selective inhibition of IL-6 oncogenic signaling on breast cancer lines.

REPORTABLE OUTCOMES

1. **Publications and abstracts.** Two manuscripts, one on MDL-16 design and the other on Evista discovery, are prepared for journal submissions. Our initial target journals are the Journal of Medicinal Chemistry and Proceedings of the National Academy of Sciences (USA). MDL-16 work was presented in the 2012 American Chemical Society Spring National meeting in San Diego, California, March 2012.
2. **Research training opportunities.** One graduate student and two postdoctoral researchers have assisted in these studies. They have been responsible for the computational, synthetic, and biological data obtained for this report. In addition, the graduate student trained in the previous 2010-2011 year, obtained her Ph.D. and joined the National Cancer Institute as a research associate.

CONCLUSIONS

In year 2 of this research project, our major progresses are 1) to discover MDL-16, a ring-opening analog of MDL-5, is better inhibitor in both potency and synthetic easiness. Careful computational analysis and alternate synthetic route confirmed these and offer insights for further optimization; 2) to discover Evista/Reloxifene, an anti-osteoposis drug, as a novel IL-6 inhibitor, opening a new lead class for inhibitor design and optimization.

The "so what section": A few of these compounds have potential as molecular probes or standards. These compounds demonstrate binding to the desired protein and inhibit STAT3 phosphorylation, something that few small molecules have been able to show.

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